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Quantitation of saxitoxin in human urine using immunocapture extraction and LC-MS

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Abstract

Aims:

An immunomagnetic capture protocol for use with liquid chromatography – tandem mass spectrometry was developed for the quantitation of saxitoxin in human urine.

Materials & Methods

This method uses monoclonal antibodies coupled to magnetic beads. Saxitoxin was certified reference material grade from National Research Council Canada. Analysis was carried out using liquid chromatography-tandem mass spectrometry.

Results

With an extraction efficiency of 80%, accuracy and precision of 93.0 – 100.2% and 5.3 – 12.6%, respectively, and a dynamic range of 1.00 – 100 ng/mL, the method is well suited to quantify STX exposures based on previously reported cases.

Conclusions

Compared to our previously published protocols this method has improved selectivity, a five-fold increase in sensitivity, and uses only one third the sample volume. This method can diagnose future toxin exposures and may complement the shellfish monitoring programs worldwide.

Keywords

Immunocapture, antibody, saxitoxin, mass spectrometry, liquid chromatography, magnetic beads, marine toxins

Introduction

Paralytic shellfish poisoning (PSP) is mainly caused by the potent neurotoxic alkaloid saxitoxin (STX) (Figure 1). PSP's primary route of exposure is through consumption of contaminated bivalve mollusks [1-4]. While PSPs can be lethal due to respiratory failure, the symptoms are generally mild and include paralysis, gastrointestinal issues, muscle weakness, and tingling or numbness of the mouth and extremities. Other than supportive care, few treatments are available for PSP [5-7]. The direct testing of patient samples for STX is the only way to truly confirm PSP toxin exposure, while also helping to avoid misdiagnosis of paralysis due to conditions with similar symptomology [8].

Traditionally, solid phase extraction (SPE) has been used extensively in the isolation of STX from various biological matrices in animals [12-15] and humans including urine, blood, and tissues [16, 17] as well as from cyanobacterium cells [18, 19]. Different formats of SPE include off-line SPE, where the isolation of analytes and analysis are carried out on different systems, and on-line SPE, where extraction and analysis of the compound of interest is completed by one combined system. SPE selectivity can be limited when the analyte and matrix components share similar physical and/or chemical characteristics. To overcome the challenges with SPE selectivity, many groups have turned to the unique properties of antibodies for sample clean up [20, 21].

The affinity of antibodies provides a unique tool to capture a molecule of interest from a complex mixture, while mass spectrometry (MS) can specifically identify and quantitate the extracted molecules. Since the foundational work by Nelson's group in 1995 [9], immunoaffinity-mass spectrometry has undergone many adaptations [10, 11], including immunomagnetic capture – liquid chromatography – tandem mass spectrometry (IMC-LC-MS). IMC-LC-MS uses magnetic

beads coated with antibody to capture target molecules from complex environmental or biological matrices. The antibody is typically chemically treated to release the target molecule for subsequent analysis using LC-MS/MS.

The use of immunocapture and mass spectrometry add specificity to the identification of STX that has not been achievable with techniques like enzyme-linked immunosorbent assays (ELISA) [22, 23], radiobinding assays (RBA) [24, 25], or electrochemical immunosensors (ECI) [26-28]. Larger molecules have been isolated using IMC, including butyrylcholinesterase nerve agent adducts [29-31], influenza proteins [32] and ricin [33]. While other works have applied IMC-LC-MS to the analysis of STX in shellfish extracts [34, 35], this is the first to use IMC-LC-MS for the small molecule STX in human matrix.

Presented here for the first time is the use of IMC-LC-MS for the detection of STX in human urine. Previous LC-MS/MS STX detection methods utilizing traditional SPE have had higher limits of detection [36, 37] or have used expensive automated on-line SPE systems [38] requiring specialized training for sample clean up. This method has reduced the complexity and sample volume requirements compared to our previously published on-line SPE method and has a lower limit of detection (LOD) compared to traditional SPE methods for STX. A lower LOD is desired since the urinary STX concentration may vary greatly based on time between exposure and collection, level of exposure, and other biological factors such as age and metabolism. In addition, a lower LOD can help to solve the problem of cases where toxins are reported in food remnants but not in patient samples. The reportable range of this method can effectively quantify expected exposures based on previously reported levels, where urine concentrations of STX have been as low as 9 ng/mL [37, 39]. Lowering the sample volume allows the method to better deal with situations where a patient is exposed to an unknown threat and the sample must be split between

multiple methods to identify the causative agent.. As the occurrences of harmful algal blooms and PSP toxins continue to increase due to pollution and climate change [40, 41] this work can facilitate the efforts of public health authorities to confirm PSP cases and may complement shellfish monitoring programs worldwide.

Materials and Methods

Chemicals, Standards, and Reagents

STX certified reference materials in 3 mM hydrochloric acid (HCl) were purchased from National Research Council Canada (Halifax, Canada). Internal standard ¹⁵N₄-labeled STX was purchased from Polysciences (Warrington, PA). Acetonitrile (ACN), methanol (MeOH), and water (all high performance liquid chromatography (HPLC) grade) were purchased from Tedia Company, Inc. (Fairfield, OH). Certified ACS Plus 12.1M HCl was purchased from Fisher Scientific (Rochester, NY). Formic acid (99%) (FA), ammonium formate (NH₄COOH), triethylammonium acetate buffer (pH 7), phosphate buffered saline with 0.5% Tween 20 (PBS-T), phosphate buffered saline (PBS), triethanolamine (TEA), dimethyl pimelimidate dihydrochloride (DMPD), and tris buffered saline (TBS) were purchased from Sigma Aldrich (Pittsburgh, PA). Deionized water (>18 MΩ·cm) was prepared on-site using an installed water purification system (Aqua Solutions, Inc., Jasper, GA). Dynabeads[®] Protein G magnetic beads (30 mg/mL), were purchased from Life Technologies (Rochester, NY). Pooled human urine and individual convenience set urine was purchased from Tennessee Blood Services (Memphis, TN). Mouse monoclonal antibody GT13-A (Ab) (1.0 mg/mL) was provided as a gift from Dr. Katrina Campbell of Queen's University Belfast.

120 *Calibrator, Internal Standard, and Quality Control (QC) materials preparation*

121 A primary stock solution of STX (1000 ng/mL) was prepared by diluting 405 µL certified STX
122 reference material (24.7 µg/mL) to 10 mL with 3 mM HCl. This solution was further diluted with
123 3 mM HCl to 100 and 10 ng/mL. These three stock solutions were then diluted with the pooled
124 human urine to prepare calibration standards at concentrations of 1.0, 2.0, 5.0, 10, 25, 50, and 100
125 ng/mL, as well as quality control (QC) samples at 7.5 and 75 ng/mL. All matrix blank samples
126 were unspiked pooled urine. The ¹⁵N₄ stock isotopically labeled internal standard was prepared
127 from the solid compound to a final concentration of 450 ng/mL in 3 mM HCl. To maintain stability,
128 all calibration standards and QCs were stored in 20 mL labeled glass vials and kept at -70°C based
129 on previous studies [37].

130 *Sample preparation*

131 *Preparation of Dynabead[®] Protein G for antibody conjugation:*

132 Dynabead[®] Protein G beads were prepared per manufacturer instructions. A 50 µL (1.5 mg) aliquot
133 of beads was washed with 100 µL PBS-T. After vortexing for 30 secs, the beads were immobilized
134 using a magnet, and the solution was removed and discarded. The beads were washed with PBS-
135 T two more times.

136 *Antibody-magnetic bead conjugation and cross-linking*

137 Mouse monoclonal antibody (22.6 µL of 1 mg/mL) and PBS (177.4 µL) were added to the washed
138 magnetic beads and incubated at room temperature at constant rotation (20 rpm) with an Invitrogen
139 Sample Mixer (Life Technologies, Rochester, NY) for 15 mins to create a final concentration of
140 15.0 µg antibody/mg beads. The antibody-beads were immobilized by placing the vial on a magnet

and the solution was discarded. Using the same process, the beads were then washed three times with 100 μ L of TEA. To cross-link the antibodies for increased stability, the beads were incubated with 5.40 mg/mL DMPD in TEA at room temperature with constant 20-rpm rotation for 30 minutes and then washed with 100 μ L of TBS. The final conjugated and cross-linked antibody-beads (Ab-bead) were placed on a magnet and were washed three times with 100 μ L PBS-T, discarding the wash solution each time.

Saxitoxin Incubation

To capture the STX from the spiked urine, 1.5 mg Ab-beads were added to 100 μ L of STX urine calibration standards, QCs, and a matrix blank. These solutions were incubated at 37°C with constant agitation at 1400 rpm for 60 mins. After incubation, the beads were immobilized with a magnet and washed three times with 500 μ L PBS, followed by a wash of 100 μ L HPLC grade water to remove any salts that might interfere with MS analysis.

Saxitoxin Extraction

The STX extraction solution was optimized by preparing three batches of Ab-bead as described above and incubating with 100 μ L of 75 ng/mL STX solution. These three batches were then extracted with 25/75, 50/50, and 75/25 ACN/2.5% formic acid. To release the STX, the Ab-beads were incubated at room temperature with a 100 μ L of the optimum 50/50 ACN/2.5% formic acid with constant agitation at 1400 rpm for 60 mins. The beads were immobilized with a magnet, and the solution containing the STX was transferred to a 96-well deep well plate. To facilitate evaporation, 100 μ L of ACN was added to the sample, and the solution was dried under nitrogen using a TurboVap (Biotage, Charlotte, NC) at 60°C and a flow rate of 60 standard cubic feet per hour (SCFH). Dried samples were reconstituted with 100 μ L of 75/25 ACN/MeOH and 6.66 μ L

internal standard, followed by vortexing at 800 rpm for 2 mins. Samples were then transferred to a 96-well autosampler plate, foil heat sealed, and analyzed by LC-MS/MS.

Chromatography and mass spectrometry conditions

Isocratic separation, with a run time of 6 mins, was carried out at a flow rate of 300 μ L/min by the Symbiosis LC system (Spark Holland, Emmen, The Netherlands) with an Atlantis Silica HILIC, 2.1 x 50 mm, 3 μ m column (Waters, Milford, MA) at 15°C. Mobile phase was comprised of 75/5/20 (v/v) ACN/MeOH/aqueous NH_4COOH (76 mM). Injection volume was 15 μ L. Positive mode turbo ion spray MS/MS with a Sciex 5500 triple quadrupole MS (Foster City, CA) was used for the detection of all analytes. Multiple reaction monitoring (MRM) transitions for STX and internal standard, along with analyte specific MS conditions, were as follows: STX, precursor ion, 300.1 m/z; product ion, 204.1 m/z (quantitation ion) and 282.1 (confirmation ion); collision energy, 25 eV and 30 eV respectively; $^{15}\text{N}_4$ -STX, precursor ion, 304.1 m/z; product ion, 207.1 m/z; collision energy, 30 eV. All of the remaining MS conditions were constant: declustering potential (DP), 45 V; entrance potential (EP), 10 V; curtain gas (CUR), 35 psi; collision gas pressure (CAD) 7 (adjusted to produce $1.7 \pm 0.1 \times 10^{-5}$ Torr); ion spray voltage, 5300 V; source temperature (TEM), 250°C (interface heater on); heater gas (GS1), 30 psi; nebulizer gas (GS2), 30 psi; cell exit potential (CEX), 23 eV; dwell time 200 msec.

Optimization of amount of antibody used for extraction

The total amount of antibody used for each extraction was optimized to maximize STX sensitivity in urine. Three different masses of Ab-beads corresponding to 11.3, 22.5, and 45 μ g of antibody were prepared and incubated with 100 μ L aliquots of 75 ng/mL STX spiked urine. After incubation and release of STX from the Ab-beads as described above, the three samples were analyzed with

LC-MS/MS and compared to a 75 ng/mL in solvent standard in 75/25 ACN/MeOH to determine recovery.

Specific vs. non-specific binding

In order to determine if the protein G beads without antibody would capture STX, two sets of beads were compared. A divided aliquot of STX in urine (200 μ L at 75 ng/mL) was incubated with the two sets of magnetic beads. The first set, representing specific binding, was prepared according to the full protocol developed here and the second set of beads was prepared in the same way but without antibody. Both samples were processed to remove the STX from the magnetic beads, analyzed by LC-MS/MS, and compared to a 75 ng/mL STX in solvent standard to determine recovery.

Extraction recovery

The effects of extraction recovery were determined in the following way. Two sets of blank pooled urine were extracted using the IMC-LC-MS method described previously. After extraction, one set of the blank urine samples were fortified with STX to a final concentration of 5 ng/mL (n=3) and the other set to 10 ng/mL (n=3). At the same time, two additional sets of urine spiked with 5 ng/mL (n = 3) and 10 ng/mL (n=3) STX were extracted in the same way as the urine blanks. After analysis, the adjusted area ratios were compared for the corresponding STX concentrations to determine the average extraction recovery [42].

206 *Incubation time study*

207 Optimal incubation time of STX with the Ab-beads was evaluated using 75 ng/mL STX in urine
208 at 15, 60, 120, and 240 mins with three replicates at each time point. Following incubation the
209 samples were removed from the mixer and treated and analyzed as described above.

210 *Background STX concentrations and spikes across the range*

211 A urine sample set of ninety-five individuals assumed to have no history of exposure to marine
212 toxins were analyzed using the IMC-LC-MS method described here. Three additional convenience
213 sample set urines were spiked to a final concentration of 7.0 ng/mL, another three samples to 30
214 ng/mL, and a final three samples to 75 ng/mL of STX. Each of the three sets of three urines were
215 from different individuals. After fortification, all samples were analyzed using the IMC-LC-MS
216 method presented here along with sets of calibration curves, QCs, and blanks.

217 *Data processing/calculations*

218 The Analyst software package (Version 1.5.2) from AB Sciex was used for the determination of
219 ion areas and linear regression analysis. Samples were quantitated using linear regression analysis
220 of the calibrator concentration versus the ratio of calibrator ion area to internal standard ion area
221 with 1/x weighting. STX was quantitated using ¹⁵N₄ labeled STX as an internal standard. . The
222 method extraction recovery of STX was calculated using the equation:

223
$$\% \text{ Extracted} = \frac{(\text{Area response of extracted STX sample})}{(\text{Area response of NMNE STX sample})} \times 100$$

224

225

3. Results and Discussion

To maximize recovery, the approach in developing the method was to optimize the amount of antibody, the STX incubation time and the solution used for extracting the STX from the antibody. This was followed by an investigation of specific versus non-specific binding. After optimization, the method performance characteristics were measured and compared to our previously developed STX methods.

Mass Spectra and Liquid Chromatography

The MS conditions used were previously developed by our group for on-line SPE analysis of STX [38]. High performance liquid chromatography tandem mass spectrometry with positive Turbo-ion spray was used for this analysis in order to provide sensitivity, selectivity, and ease of quantitation. After comparison of multiple columns, an Atlantis HILIC column was selected as optimum based on better peak shape and shorter retention time. A flow rate of 300 μ L/min and a mobile phase of 75/5/20% ACN/MeOH/ NH_4COOH were used for all separations with a column temperature and injection size of 15°C and 15 μ L, respectively. The quantitation and confirmation transitions chosen were 300.1->204.1 and 300.1->282.1, respectively.

Optimization of amount of antibody for immunocapture

The amount of antibody needed to extract STX from urine was first optimized with a recovery of 81%, similar to the official STX AOAC 2005.06 method for mussel samples [43]. Three different masses of antibody, with a fixed amount of beads (1.5 mg), were evaluated, 11.3, 22.5, and 45.0 μ g. The 11.3 μ g resulted in 68% recovery, while the 22.5 μ g and 45.0 μ g of antibody both gave 81% recovery. The 22.5 μ g of antibody was chosen for further experiments in order to maximize recovery while not wasting Ab-beads. The labeled and unlabeled saxitoxin may compete for the

same binding sites of the antibody leading to a need for more antibody and increasing the method cost. This is alleviated by adding the internal standard after extraction. .

STX Extraction

Using solutions of 25/75, 50/50, and 75/25 ACN/2.5% formic acid, three different batches of Ab-beads were extracted to determine the optimum STX release solution. After comparison to a non-extracted STX sample at the same concentration the percent recovery was determined for each batch. The 25/75 solutions had a recover of 56%, while the 75/25 solutions recovery was 62%. With a recovery of 80%, the 50/50 ACN/2.5% formic acid solution was chosen as optimum for the release of STX from the Ab-bead.

Specific vs non-specific binding

Specific versus non-specific binding of STX was investigated to determine if the protein G bead could capture STX without antibody attached (Figure 2). Previous work in our group showed that some compounds could bind directly to the bead without the need of antibody. For this reason, this comparison of specific and non-specific binding was explored. The magnetic beads with antibody showed significantly higher recovery as compared to those without antibody (80% vs. 2% recovery, respectively) (Figure 2). While the protein G beads can capture a small amount of STX from urine, the 2% would not be capable of capturing enough analyte for analysis, especially at lower concentrations. Also, the addition of the antibody to the protein G on the bead surface should occlude any unreacted protein G sites and reduce the chances of any non-specific binding.

STX incubation time study

To determine the optimum incubation time between sample and Ab-beads for maximum extraction recovery four time points (15, 60, 120, and 240 mins) were explored (Figure 3). While the 15 minute incubation resulted in a $45 \pm 5\%$ recovery of STX, the other three incubation times all resulted in approximately 80% recovery (60 min: $79 \pm 4\%$; 120 min: $81 \pm 5\%$; 240 min: $75 \pm 6\%$). Sixty minutes was selected as the ideal incubation time to minimize sample preparation time while maintaining relatively high extraction recovery.

Method performance characteristics

Method characterization was based on the guidelines presented in the Division of Laboratory Sciences Policies and Procedures Manual [44]. Ten sets of calibration standards and QCs were prepared according to the final method protocols determined above. These sets were analyzed over the course of a month with no more than two curves being extracted and analyzed each day. Two matrix blank unfortified urine samples were also analyzed with each calibration curve as negative QCs. Regression analysis showed a linear fit of the data is acceptable based on the random distribution of residual values about the x-axis (Figure 4A) and an average coefficient of determination (R^2) value of 0.9930 ($n = 10$). The low QC (7.5 ng/mL) had an accuracy of 97.4% with an 11.3% RSD, while the high QC (75 ng/mL) had an accuracy of 94.6% with a 12.6% RSD. All accuracy and precision values were within the range recommended by the Food and Drug Administration (Table 1) [45]. There were no detectable signals for the ninety-five convenience urine samples analyzed or matrix blanks, which reflects that this is a highly selective diagnostic test for STX exposure (Figure 4B).

The lowest reportable limit for the method was set at the lowest calibrator (STX concentration 1.00 ng/mL). The Taylor method was used to determine LOD by calculating the standard deviation of the three lowest calibrators following 10 repeated measurements [46]. The standard deviations were then plotted versus theoretical concentrations, and the intercept of the least squares regression analysis determined the standard deviation of the blank, S_0 . The LOD was then calculated as $3S_0$, which was 0.526 ng/mL for STX. Analysis of the convenience sample set showed no detectable presence of saxitoxin in any unspiked urine. The additional nine convenience samples spiked at 7.0 ng/mL (n=3), 30 ng/mL (n=3), and 75 ng/mL (n=3), had accuracies between 88% and 90%, and precisions between 5.6% and 13% (Table 1).

Finally, for method evaluation, ion ratios were calculated using the areas of the quantitation and confirmation transitions of 300.1 \rightarrow 204.1 and 300.1 \rightarrow 282.1, respectively. The ion ratios of the STX QCs and calibrators during method characterization averaged 0.62 with a %RSD of 12. The confirmation ion was detected at the lowest calibrator where it averaged 0.63 with a %RSD of 13% and was within 9% for all fortified STX samples.

Comparison of on-line, off-line and immunomagnetic separation methods

As a final step in the proof of concept, the new IMC-LC-MS method was evaluated against the methods previously reported by our group for STX analysis in urine. Both of our previously developed methods utilize SPE. As shown in Table 2, the IMC-LC-MS method had a lower LOD of 1.0 ng/mL, as compared to 4.8 ng/mL for the off-line method. Precision and accuracy were $\pm 15\%$ across the range, while requiring 30 – 50% less sample volume. One limitation of the new method compared to the on-line method was the shorter dynamic range. For concentrations greater than 100 ng/mL STX, the IMC-LC-MS method resulted in a non-linear response. This may be

caused by over saturation of the detector or over saturation of the Ab-bead. Another cause maybe that the method was optimized at 75 ng/mL STX instead of at a concentration above 100 ng/mL. Additionally we realized late in the method development that we were exceeding the binding capacity of the protein G bead by more than double. While this did ensure full coverage of the bead with antibody, it waste antibody and will be corrected in future development of this method. Even with the difference in dynamic range, this method can measure clinically significant exposures that have been seen as low as 9 ng/mL or more [37, 39]. In addition to having an acceptable dynamic range with accuracy and precision within $\pm 15\%$, the IMC-LC-MS method has greater selectivity eliminating most of the background interference peaks from the matrix. Figure 5 shows an overlay of a 75 ng/mL urine calibrator analyzed using our previously developed on-line SPE method and our new IMC-LC-MS method. The background peaks between 1.5 and 3 mins are completely removed and those after 5 mins are reduced by an order of magnitude.

Conclusion

Compared to our previously published methods, this IMC-LC-MS analysis for the detection of STX in human urine has improved selectivity compared to our online SPE method and a five-fold increase in sensitivity compared to our offline method. Compared to both off-line and on-line SPE methods it uses less sample volume for extraction. Using less sample volume means this method is suited for situations where sample is and must be split between multiple methods. This method can effectively quantify exposure to PSP toxins based on previously reported suspected exposure victims [37, 39]. In addition, compared to the on-line SPE method it reduces the amount of matrix interference peaks seen across the elution window. This work will provide a complimentary method to the ongoing analysis of environmental samples. In addition, this method is a stepping-

stone to expanding the specific identification of other poisons and toxins. Antibodies have been developed that bind other marine toxins such as domoic acid [47], the gonyautoxins [48], and tetrodotoxin [49]. These antibodies can be incorporated into the present method by adding additional magnetic beads with the conjugated antibodies to the samples. Using this technique multiple toxins with different chemical properties could be extracted from a single aliquot, something generally not achievable with traditional SPE. With the appropriate approvals, this method could be applied to real world exposure samples where the causative toxic agent is unknown, a limitation of the current method.

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Figures and Tables

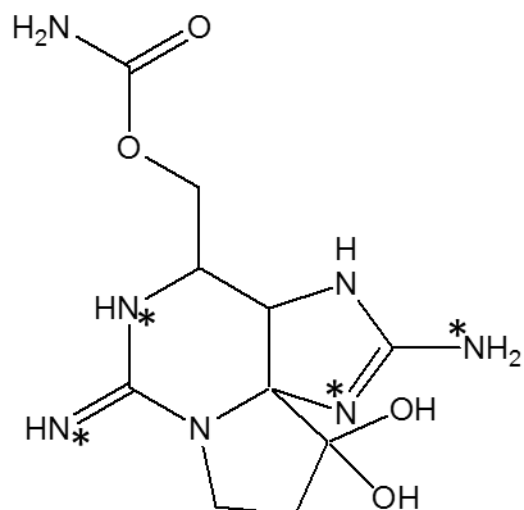


Figure 1: Chemical structure of saxitoxin. The * denote the ^{15}N labels of the internal standard.

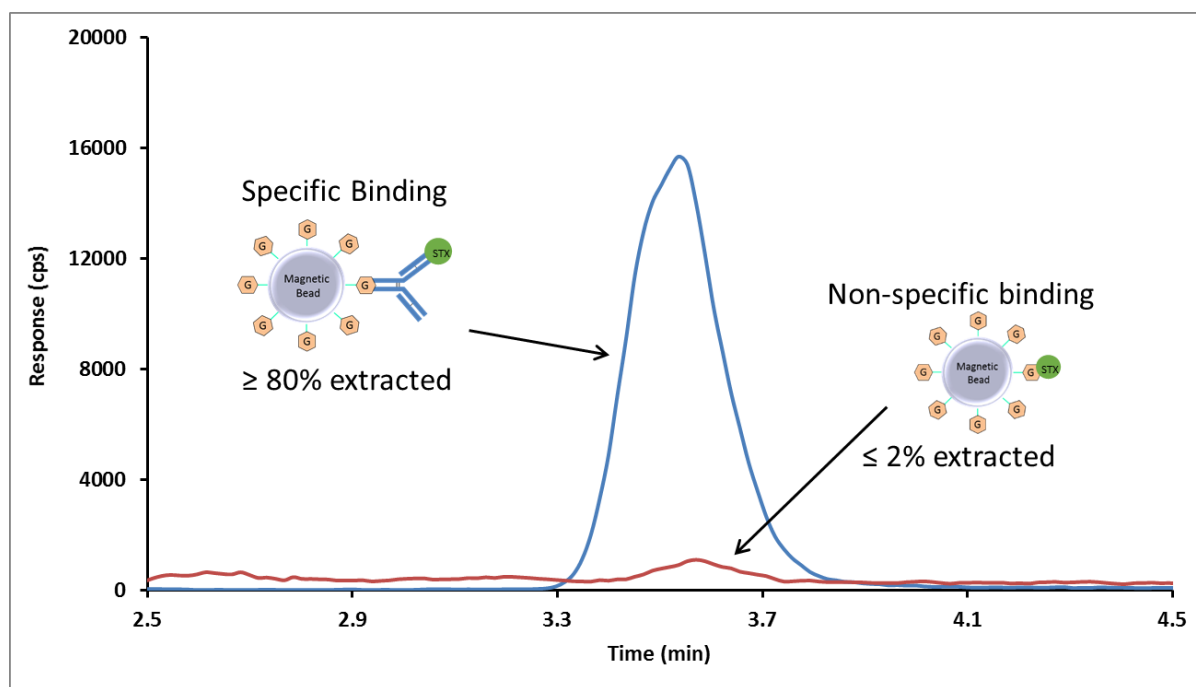


Figure 2: Comparison of specific binding of STX to the antibody-bead conjugate and non-specific binding of STX to the protein G magnetic bead alone. For percent extracted $n = 3$.

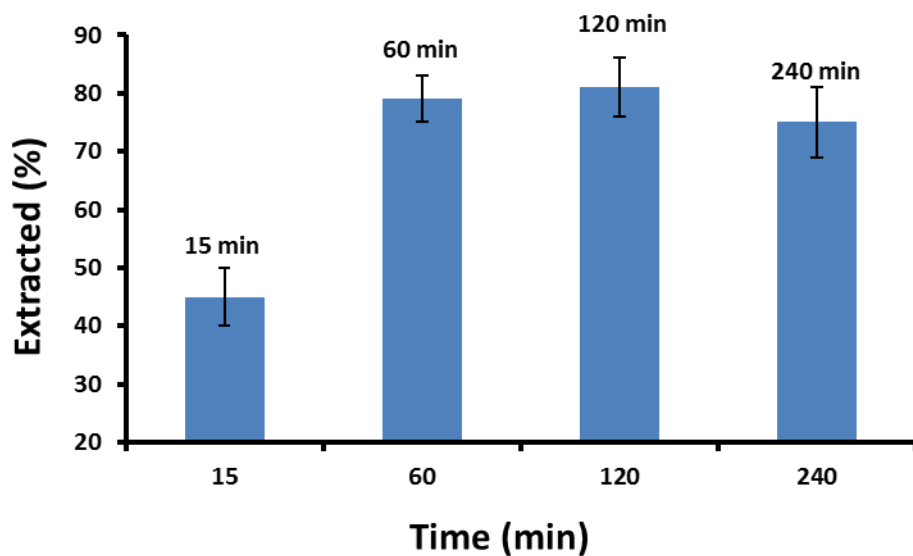


Figure 3: Comparison of incubation time of STX with antibody coated beads. Error bars represent the percent relative standard deviation (n = 3).

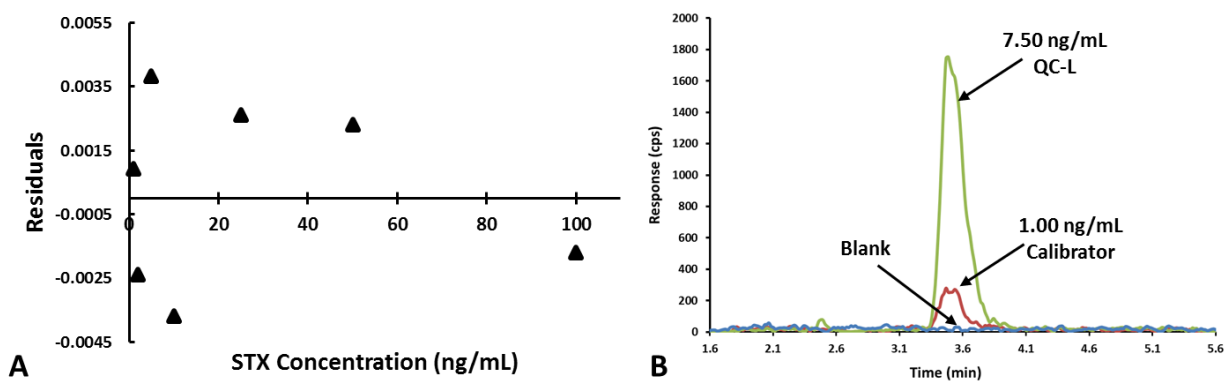


Figure 4: A. Residuals analysis of STX calibrators 1.0, 2.0, 5.0, 10, 25, 50, and 100 ng/mL B. Comparison of chromatograms of matrix blank, 1 ng/mL calibrator, and low 7.5 ng/mL QC-L.

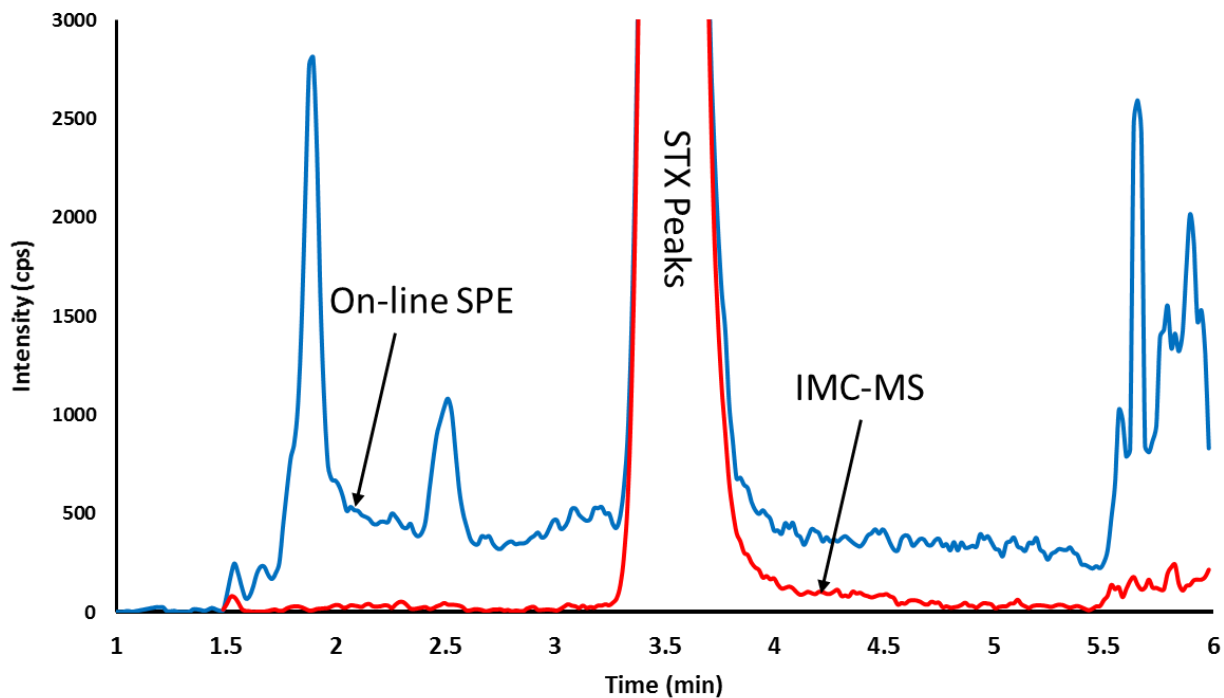


Figure 5: Overlay comparing 75 ng/mL urine calibrator analyzed using on-line SPE method and IMC-LC-MS method.

Table 1: Summarized method performance characteristics for calibrators and QCs (n=10) and spiked urine samples across the dynamic range (n=3).

Calibrators & QCs	Theoretical Concentration (ng/mL)	Mean (ng/mL)	Average Accuracy	Average Precision
Calibrator 1	1.00	0.951	95.1%	12.6%
Calibrator 2	2.00	1.89	94.5%	10.8%
Calibrator 3	5.00	5.01	100.2%	9.50%
Calibrator 4	10.0	9.87	98.7%	11.3%
Calibrator 5	25.0	24.8	99.2%	10.2%
Calibrator 6	50.0	46.5	93.0%	5.40%
Calibrator 7	100	94.2	94.2%	7.80%
QC-L	7.50	7.21	97.4%	11.3%
QC-H	75.0	71.8	94.6%	12.60%
Spiked Individual Urine	Theoretical Concentration (ng/mL)	Mean (ng/mL)	Average Accuracy	Average Precision
Spike 1	7.00	6.15	87.9%	5.60%
Spike 2	30.0	26.9	89.7%	13.0%
Spike 3	75.0	67.5	90.0%	6.80%

622 **Table 2:** Comparison of method characteristics for off-line (n = 20), on-line (n = 20), and IMC-
623 LC-MS (n = 10) methods for the extraction and analysis of STX in human urine.

	Off-line SPE	On-line SPE	IMC-MS
Accuracy QC-L	94 ± 6.1%	97 ± 4.2%	95 ± 7.2%
Accuracy QC-H	99 ± 4.5%	103 ± 5.4%	96 ± 7.1%
Reportable range	4.8 - 145 ng/mL	1.0 - 150 ng/mL	1.0 - 100 ng/mL
Intraday %RSD	-	0.08 - 4.2%	3.3 - 9.6%
Sample volume	1125 µL	300 µL	100 µL

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